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ABSTRACT Mitochondrial 16s rDNA was sequenced from nine different populations of Aphelinidae and 10 of aphidiinae. Sequence divergences between populations within a species are low, ranging from 0 to 0.38%. Divergences among species within the same genus range from 0 to 8.71%. *Aphelinus asychis* Walker has a sequence divergence from the other *Aphelinus* spp. of 8.71%, which is even higher than the divergences among the aphidiine genera *Lysiphlebus*, *Aphidius* and *Diaeretiella*. Our 16s rDNA phylogeny for aphidiines is concordant with a previously published NADH1 dehydrogenase phylogeny. Our analysis identifies a complex comprising *A. albipodus* (Hayat & Fatima), *A. varipes* (Foerster), and *A. hordei* (Kurdjumov). The sequence divergences within this complex are low, ranging from 0 to 0.38% with an average of 0.19%, which suggests that the three species within the *A. varipes* complex diverged within the last ≈90,000 yr.

KEY WORDS *Aphelinus*, Aphelinidae, Braconidae, aphidiinae, 16s rDNA, phylogeny

THE MINUTE PARASITOIDS of the genus *Aphelinus* are important biological control agents of aphids worldwide. During the last four decades, four species, *A. albipodus* (Hayat & Fatima), *A. asychis* (Walker), *A. varipes* (Foerster), and *A. hordei* (Kurdjumov), have been introduced into the United States to control several aphid species (van den Bosch et al. 1959, Jackson et al. 1970, Jackson and Eikenbary 1971, Hopper et al. 1998, Prokrym et al. 1998a, Zhu et al. 2000). Additional taxa, which may be synonymous with one or more of these species, have been reported from North America (Mackauer and Finlayson 1967, Lajeunesse and Johnson 1991, Yu 1992). Also, the *A. asychis* individuals released in the United States comprise a group of geographical isolates that may constitute a sibling species complex (Kazmer et al. 1996). These difficulties in determining taxonomic and geographic affinities of aphelinids exerting cereal aphid biological control make it difficult to evaluate the effectiveness of the biological control programs through which they were introduced (Hopper et al. 1998, Prokrym et al. 1998a).

The taxonomy of these species is also made difficult by their small size (≤1.5 mm long) and the paucity of clear morphological differences among them (Prokrym et al. 1998b). Several biochemical approaches, including allozyme electrophoresis (Walton et al. 1990), random amplified polymorphic DNA markers (Kazmer et al. 1995), microsatellite DNA variability (Vanlerberhe-Masutti and Chavigny 1997), and ITS-2 polymerase chain reaction (PCR) (Zhu and Greenstone 1999, Zhu et al. 2000), have been used to differentiate *Aphelinus* spp.

Ribosomes are a part of the translational machinery of the cell. Thus, the sequences and structures of rRNAs are conserved in the course of evolution due to their importance in the growth, function and survival of cells (Gutell 1992). Classical analysis of complete and partial sequences of rRNA has revealed that the primary structure of rRNAs consists of highly conserved regions interspersed with regions of moderate to low homology within related species (Gopo et al. 1988). The small size of 5S rRNA and extensive secondary structures in the 23S rRNA render these molecules unsuitable for sequencing and further analysis. However, the 16s rRNA has been found to be specific to a given species. The moderate size of the 16s rRNA, range of evolutionary rates across the sequence, and confirmed importance in systematics make 16s rRNA particularly useful for phylogenetic studies (Simon et al. 1994, Raghava et al. 2000).

As part of an ongoing program to develop molecular markers to separate and identify aphid endoparasitoids (Zhu and Greenstone 1999, Zhu et al. 2000), we have sequenced a segment of the mitochondrial 16s rDNA gene for these four *Aphelinus* species. For comparative purposes, we also sequenced a less problem-

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Table 1. Taxa included in the study

Species name	abbreviation	Locality or reference
Aphelinus asychis Walker	aaska	Kazakhstan
Aphelinus asychis Walker	aasmp	Montpellier, France
Aphelinus albipodus Hayat & Fatima	aal	Tacheng, China
Aphelinus varipes (Foerster)	avalj	Lattes-Juvignac, France
Aphelinus varipes (Foerster)	avamp	Montpellier, France
Aphelinus hordei (Kurdjumov)	aho	Odessa, Ukraine
Aphytis melinus DeBach	ame	Dowton & Austin 1994
Encarsia formosa Gahan	efo	Dowton & Austin 1994
[a]*Pteromalus puparum* (L.)	ppu	Dowton & Austin 1994
Diaeretiella rapae (M'Intosh)	draco	Colfax, WA, USA
Diaeretiella rapae (M'Intosh)	drauc	Riverside, CA, USA
Lysiphlebus testaceipes (Cresson)	ltesw	Stillwater, OK, USA
Lysiphlebus testaceipes (Cresson)	ltepu	Pullman, WA, USA
Lysiphlebus testaceipes (Cresson)	ltech	Chelan, WA, USA
Lysiphlebus testaceipes (Cresson)	lteel	Ellensburg, WA, USA
Aphidius colemani Viereck	acoch	Chile
Aphidius colemani Viereck	acook	Stillwater, OK, USA
Aphidius ervi Haliday	aer	Prosser, WA, USA
Aphidius matricariae Haliday	ama	Prosser, WA, USA
Aphidius ervi Haliday	aergb	Belshaw et al. 2000
Aphidius rosae Haliday	aro	Dowton et al. 1998
Pseudephedrus chilensis (Howard)	pch	Belshaw et al. 2000
Dyscritulus planiceps (Marshall)	dpl	Belshaw et al. 2000
Ephedrus plagiator (Nees)	epl	Belshaw et al. 2000
Paralipsis enervis (Nees)	pen	Belshaw et al. 2000
Lipolexis gracilis	lgr	Kambhampati et al. (GenBank accession No. AF174338)
[a]*Hormius* sp.	hsp	Belshaw et al. 2000

[a] *Pteromalus puparum* was used as outgroup for Aphelinidae, and *Hormius* sp. for Aphidiina.

atic group of aphid parasitoids of the braconid subfamily aphidiinae (GenBank accession numbers AF289130 to AF289148). Here we present phylogenies based on these sequences, and use estimates of relationships and divergence times among taxa to infer the systematic status of the aphelinid taxa.

Materials and Methods

Insects. Most of the sequences used in this research were derived from parasitoids that were either received preserved in 95% ethanol or were removed from established colonies in the laboratory; additional sequences were derived from the literature. Colonies were reared by previously described protocols (Reed et al. 1991), and maintained in cages in a Conviron model I23 incubator (Controlled Environments, Pembina, ND) at 20°C and a photoperiod of 16:8 (L:D) h. To reduce the risk of contamination, only one parasitoid colony was maintained at one time. The collecting localities or literature sources of all populations used in this research are given in Table 1.

DNA Extraction. We modified the methods of Zhu and Greenstone (1999) to extract total DNA. Insects were placed individually in 1.5-ml microcentrifuge tubes and homogenized using a battery-powered homogenizer (Midwest Scientific, St. Louis, MO) in 100 μl of isolation buffer containing 0.1 M NaCl, 0.2 M sucrose, 0.1 M Tris-HCl (pH 9.1), 0.05 M EDTA, 1% SDS, and 20 μg/ml RNase A. The homogenate was vortexed briefly and incubated for 30 min at 65°C. The supernatant was transferred to a new tube and extracted once with one volume of chloroform/isoamyl alcohol (24:1). One-tenth volume of 3.0 M sodium acetate and two volumes of ice-cold 100% EtOH were added to the tube. DNA was then pelleted by centrifugation and resuspended in 200 μl of distilled water.

PCR Amplification, Purification, and Sequencing of 16s rDNA. Portions of the mitochondrial 16s rDNA gene were amplified by PCR using primers designed by Dowton and Austin (1994). The primers used were 16SWb (5′-CACCTGTTTATCAAAAACAT-3′), which anneals to nucleotides 13924–13943, and 16SWa (5′-CGTCGATTTGAACTCAAATC-3′), which anneals to nucleotides 13392–13411 of the honey bee mitochondrial genome (Crozier and Crozier 1993). Where amplifications were unsuccessful under a variety of conditions, the primer 16S.Sh (5′-AGATTTTAAAAGTCGAACAG-3′), which anneals to nucleotides 13417–13436 of the honey bee genome, was used in place of 16SWa. The PCR product from the 16s rDNA gene was thus in the range of 530–550 bp.

PCR reactions (25 μl) contained 10 mM Tris-HCl pH 9.0, 1.5 mM $MgCl_2$, 1.0 μM of each primer, 50 mM KCl, 0.1 mM of each dNTP, 0.05 U/μl of *Taq* DNA polymerase (Promega, Madison, WI), and 2 μl of DNA template, and were performed in a PTC-100 thermocycler (MJ Research, Watertown, MA). DNA was denatured for 3 min at 94°C, followed by 35 amplification cycles comprising 30 s denaturing at 94°C, 30 s annealing at 50°C, and 1 min extension at 72°C. DNA was finally extended for 2 min at 72°C after amplification.

Table 2. 16s rDNA sequence divergence (%) (above the diagonal) and number of differences (below the diagonal) based on pairwise comparisons among aphid parasitoid populations

	ltepu	ltech	lteel	ltesw	drauc	draco	acoch	acook	aer	ama	aaska	aasmp	avalj	avamp	aho	aalca
1. ltepu		0	0	0.38	4.58	4.58	5.17	5.17	5.56	5.75	24.38	24.38	25.24	25.24	25.05	25.05
2. ltech	0		0	0.38	4.58	4.58	5.17	5.17	5.56	5.75	24.38	24.38	25.24	25.24	25.05	25.05
3. lteel	0	0		0.38	4.58	4.58	5.17	5.17	5.56	5.75	24.38	24.38	25.24	25.24	25.05	25.05
4. ltesw	2	2	2		4.96	4.96	5.53	5.53	5.94	6.13	24.38	24.38	25.19	25.19	25.38	25.38
5. drauc	24	24	24	26		0	3.26	3.26	2.68	3.26	24.00	24.00	25.05	25.05	24.86	24.86
6. draco	24	24	24	26	0		3.26	3.26	2.68	3.26	24.00	24.00	25.05	25.05	24.86	24.86
7. acoch	27	27	27	29	17	17		0	3.64	4.41	24.57	24.57	25.05	25.05	24.86	24.86
8. acook	27	27	27	29	17	17	0		3.64	4.41	24.57	24.57	25.05	25.05	24.86	24.86
9. aer	29	29	29	31	14	14	19	19		1.72	24.57	24.57	26.01	26.01	25.82	25.82
10. ama	30	30	30	32	17	17	23	23	9		25.05	25.05	26.40	26.40	26.20	26.20
11. aaska	127	127	127	127	126	126	128	128	128	130		0.19	8.71	8.71	8.71	8.71
12. aasmp	127	127	127	127	126	126	128	128	128	130	1		8.71	8.71	8.71	8.71
13. avalj	131	131	131	132	130	130	130	130	135	137	46	46		0	0.38	0.19
14. avamp	131	131	131	132	130	130	130	130	135	137	46	46	0		0.38	0.19
15. aho	130	130	130	133	129	129	129	129	134	136	46	46	1	1		0
16. aal	130	130	130	133	129	129	129	129	134	136	46	46	1	1	0	

PCR products were separated on a 1% low melting point agarose gel. DNA fragments were sliced from the gel and extracted using a wizard PCR Preps DNA Purification System (Promega). Purified DNA fragments were directly sequenced from both directions using an automated sequencer located at the Recombinant DNA/Protein Resource Facility, Oklahoma State University, Stillwater.

Phylogenetic Analysis. The amplified 16s rDNA sequences were confirmed by similarity searching of the GenBank database using the Fasta and Blast programs in the GCG Wisconsin Package. We aligned 16s rDNA sequences by using Seqlab and Pileup of the GCG Wisconsin Package (Unix version 10, Genetics Computer Group, Madison, WI), and confirmed alignment by visual inspection. MEGA program version 1.01 (Kumar et al. 1993) was used to estimate evolutionary distances and compute basic statistical quantities.

Phylogenetic analyses were done using neighbor-joining, parsimony, and maximum likelihood methods with the software PAUP (Swofford 1997). *Pteromalus puparum* (L.) (Hymenoptera: Pteromalidae) was used as the outgroup for Aphelinidae, and *Hormius* sp. (Hymenoptera: Braconidae) for aphidiinae. To find minimum-length phylogenetic trees, heuristic parsimony searches (Hillis et al. 1996) were performed using 100 replicates of random additions with the tree bisection and reconnection option for branch swapping. The stepwise addition option was used to create an initial tree for the heuristic search. Random number seed was reset for random resolution of polytomies during the swapping procedure. Maximum likelihood trees using nucleotide data were generated under the Hasegawa-Kishino-Yano substitution model (Hasegawa et al. 1985) using observed base frequencies. Transition/transversion ratios estimated by PAUP were used in maximum likelihood analyses. Heuristic searches were used with 10 times of random additions and the tree bisection and reconnection option. To assess the support of clades in the phylogenetic trees, the bootstrap test (Felsenstein 1985) was performed with 1000 replications. The trees were visualized by Treeview (version 1.6.1; Page 1996).

Voucher Specimens. Voucher specimens of *A. hordei* and all of the aphidiine species have been deposited in the USDA-ARS-PSWCRL Cereal Insect Genetics Resource Library, Stillwater, OK. Voucher information for the other aphelinid species was reported previously (Zhu and Greenstone 1999).

Results

Sequence Data and Divergence. The 16s rDNA PCR products ranged from 524 to 531 bp; the aligned sequences were 534 bp long due to the introduction of gaps among sequences. Of 534 total characters analyzed, 363 (67.98%) are constant, and the number of parsimony-informative characters is 165 (30.90%). For the *Aphelinus* spp., 340 (63.43%) characters are constant and the number of parsimony-informative characters is 84 (15.67%); for the aphidiines, 164 characters are constant (30.83%), and the number of parsimony-informative characters is 137 (25.75%).

Sequence divergences differ greatly between different pairs of taxa, ranging from 0 to 26.4% (Table 2). Divergences within species are generally low. For four populations of *Lysiphlebus testaceipes* (Cresson), the sequence divergence ranges from 0 to 0.38%. No sequence differences were found within populations of *Diaretiella rapae* (M'Intosh), *Aphidius colemani* Viereck, and *A. varipes.* The sequence divergence between the two populations of *A. asychis* is 0.19%Sequence divergence among congeneric species is generally higher than within species, ranging from 0 to 8.71%. The divergence among the three *Aphidius* species ranges from 1.72 to 4.41%. The sequence of *A. hordei* differs from that of *A. varipes* by 0.38% and from that of *A. albipodus* by 0.19%.

Phylogenetic Relationships. For the aphelinids, six equally parsimonius trees were constructed and from these a consensus tree, using *P. puparum* as the outgroup, was generated (Fig. 1). All *Aphelinus* species

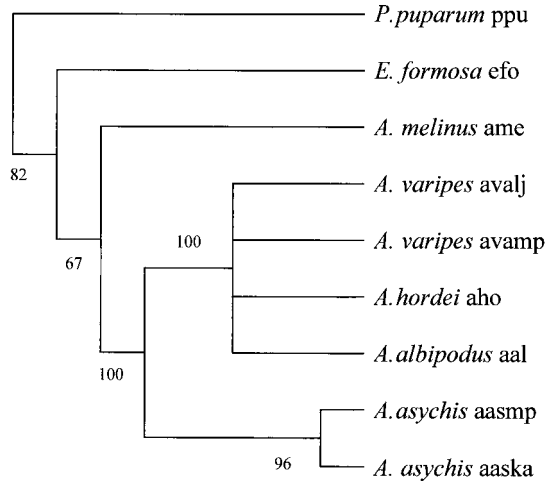

Fig. 1. Strict consensus of six equally parsimonious trees resulting from maximum parsimony analysis for aphelinids. Of 536 total characters, 340 characters are constant; 84 are parsimony-informative. Tree length = 249, consistency index (CI) = 0.8394, retention index (RI) = 0.7546, rescaled consistency index (RC) = 0.6334. Numbers at branches are bootstrap values with 1,000 bootstrap replications. Abbreviations as in Table 2.

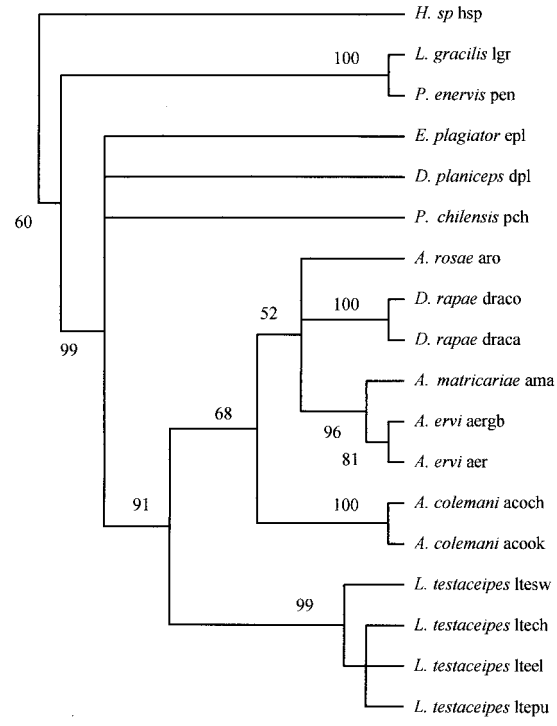

Fig. 2. Single most parsimonious tree yielded by maximum parsimony analysis for aphidiine wasps. Of 532 total characters, 164 are constant, 137 are parsimony-informative. Tree length = 644, CI = 0.7966, RI = 0.6042, RC = 0.4813. Numbers and abbreviations as in Fig. 1.

form a monophyletic group with 100% bootstrap value support. Within *Aphelinus*, two clades are obvious, one with two *A. asychis* strains and the other containing the other three species, both with 100% bootstrap value support. These three species are shown as monophyletic.

For aphidiine wasps, a single most parsimonious tree, using *Hormius* sp. as the outgroup, resulted from the parsimony analysis (Fig. 2). All species and strains of *Lysiphlebus*, *Diaeretiella* and *Aphidius* form a monophyletic group. *Diaeretiella* and *Aphidius* are more closely related to each other than to *Lysiphlebus*. *Aphidius* is paraphyletic due to the inclusion of *Diaeretiella*.

For both aphelinids and aphidiines, trees obtained from neighbor-joining and maximum likelihood analyses were very similar to those from the parsimony analysis (data not shown). This indicates strong support for the clades.

Discussion

Among the parasitoid genera studied here, sequence divergences are high, ranging from 2.68 to 26.4%. *Aphelinus* has the highest sequence divergence, >20% from the other genera. The sequence of *A. asychis* is very different from those of its congeners, diverging from them by 8.71%, which is even higher than the divergence among the aphidiid genera *Lysiphlebus*, *Aphidius*, and *Diaeretiella*, which range from 2.68 to 6.13%. The 16s rDNA sequences of *A. varipes*, *A. hordei*, and *A. albipodus* are sufficiently similar to suggest that they belong either to the same species or to a species complex distinguishable from *A. asychis*. Here we conservatively consign all of the individuals from the *A. albipodus*, *A. varipes*, and *A. hordei* populations studied here to an *A. varipes* species complex.

Our aphidiine phylogeny supports the NADH 1 dehydrogenase gene phylogeny of Smith et al. (1999), in which *Diaeretiella* also falls inside *Aphidius*. *Aphidius ervi* Haliday and *A. matricariae* Haliday are closely related to one other, and *A. colemani* is a basal taxon within the *Aphidius* and *Diaeretiella* complex. A 28S D2 phylogeny (Belshaw et al. 2000) indicated that the genus *Lysiphlebus* is polyphyletic.

If we assume a rate of divergence for mtDNA of 2% per million years (Myr) as in *Drosophila* (DeSalle et al. 1987), we find that the average sequence divergence between *A. asychis* and the members of the *A. varipes* complex corresponds to a time of 4.36 Myr. Using the same rate, the average sequence divergence within the *A. varipes* complex, 0.19%, corresponds to only 90,000 yr. The lack of sequence variation in 16s rDNA between *A. hordei* and *A. albipodus* may indicate that they belong to the same species.

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